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The mosquito *Aedes aegypti* (L.) leucokinin receptor is a multiligand receptor for the three *Aedes* kinins

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Abstract

A cDNA cloned from *Aedes aegypti* (L.) (Aedae) female Malpighian tubule (AY596453) encodes a 584 amino acid residue protein (65.2 kDa) predicted as a G protein-coupled receptor and orthologue of the drosokinin receptor from *Drosophila melanogaster* and highly similar to the tick *Boophilus microplus* myokinin receptor (AF228521). Based on the similarity to this *Aedes* sequence, we also propose a correction for the *Anopheles gambiae* protein sequence EAA05450. When expressed in CHO-K1 cells, the *Aedes* receptor behaved as a multiligand receptor and functionally responded to concentrations ≥ 1 nM of Aedae kinins 1–3, respectively, as determined by a calcium bioluminescence plate assay and single cell intracellular calcium measurements by confocal fluorescence cytometry. Estimates of EC_{50} values by the plate assay were 16.04 nM for Aedae-K-3, 26.6 nM for Aedae-K-2 and 48.8 nM for Aedae-K-1 and were statistically significantly different. These results suggest that the observed differences in physiological responses to the three *Aedes* kinins in the *Aedes* isolated Malpighian tubule reported elsewhere could now be explained by differences in intracellular signalling events triggered by the different peptides on the same receptor and not necessarily due to the existence of various receptors for the three *Aedes* kinins.

Keywords: insect GPCR (G protein-coupled receptor) (myo)kinin receptor, insect diuresis, leucokinin(s), insect excretion.

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Introduction

In female mosquitoes that are vectors of disease, such as those from *Aedes aegypti* and *Anopheles gambiae*, blood feeding is required for oogenesis; it triggers vitellogenesis necessary for egg development. The female of *A. aegypti* ingests about twice its weight in blood during feeding (Stobbart, 1977) and about 40% of the water ingested with the blood meal is excreted in the first hour after feeding; the rest remains in the plasma contained within the midgut (Williams *et al*., 1983). Most of the water load is secreted by the Malpighian tubules into the hindgut that excretes it. As insects do not have a high-pressure circulatory system, urine formation occurs by the hormonal control of ion transport. Ion gradients are established in the Malpighian tubule and water follows (reviewed in Coast *et al*., 2002). In the female mosquito urine is formed by the secretion of NaCl and KCl; Na^+ and K^+ are secreted from the haemolymph against their electrochemical potentials, their transport energized by the V-ATPase in the apical membrane of principal cells, while Cl^- moves from the haemolymph into the tubule lumen down its electrochemical potential (passive transport) (Beyenbach, 2003a).

In culicid mosquitoes diuretic hormones acting on the Malpighian tubule control the formation of urine (Wheelock *et al*., 1988); diuresins and leucokinins are involved. The mosquito natriuretic peptide (MNP), probably a corticotropin-releasing factor-like peptide (diuresin), is released from the head (Petzel *et al*., 1985, 1987). In the genome of *An. gambiae* two diuresin genes have been identified (Riehle *et al*., 2002) as well as two putative diuresin receptors (Hill *et al*., 2002). The leucokinins, first discovered as myotropic in the cockroach *Leucophaea maderae*, and leucokinin-like neuropeptides (*Aedes* kinins) also increase fluid secretion in the *A. aegypti* isolated Malpighian tubule (for a review see Beyenbach, 2003b). In *A. aegypti*, correspondingly, the excreted urine is enriched in Na^+ and Cl^- during the peak of diuresis that lasts 10 min following the onset of the blood meal (Petzel *et al*., 1985). The MNP (and cAMP) regulates the transcellular transport of Na^+ while the *Aedes* kinins increase the passive Cl^- conductance through the paracellular route (Wang *et al*., 1996). The (leuco)kinin family is characterized by the C-terminal pentapeptide sequence Phe–Xaa1–Xaa2–Trp–Gly-amide that is the minimal required for activity

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(Xaa1 can be Asn, His, Phe, Ser or Tyr and Xaa2 can be Ser, Pro or Ala). For a recent review on kinins see Torfs *et al*. (1999). The nomenclature of peptides and receptors used is as proposed by Coast *et al*. (2002). Leucokinins were first found to have diuretic activity on isolated Malpighian tubules of *A. aegypti* females (Hayes *et al*., 1989). Subsequently, of the three endogenous *Aedes* kinins (Aedae-K), the Aedae-K-1 and -3 were shown to have diuretic activity *in vitro* (Veenstra *et al*., 1997). Aedae-K-2 causes a depolarization of the transepithelial voltage but has no significant effect on fluid secretion *in vitro* (Veenstra *et al*., 1997). More recently, it was shown that both diuresin (from *Culex salinarius*) and the three *Aedes* kinins, when injected in *A. aegypti* females, increase urine production in a dose-dependent manner, supporting their role as hormones *in vivo* (Cady & Hagedorn, 1999b). About thirty-seven leucokinin-like peptides have been isolated from invertebrates. Leucokinins activate G protein-coupled receptors (GPCRs) that transduce the hormonal signal through heterotrimeric guanine nucleotide-binding proteins (G proteins) through an increased production of IP_3, causing the release of calcium from intracellular stores through the IP_3 receptor (Radford *et al*., 2002). For a recent review on invertebrate GPCRs, including kinin receptors, see Vanden Broeck (2001). In the isolated Malpighian tubule of *A. aegypti*, the three *Aedes* kinins increase intracellular IP_3 (Cady & Hagedorn, 1999a) and elicit Ca^{2+} release from intracellular stores in principal cells (Yu & Beyenbach, 2002); diuresins increase cAMP (Cady & Hagedorn, 1999a).

The leucokinin receptor has not yet been cloned from mosquitoes. We previously identified a 54–62 kDa leucokinin-binding protein with receptor characteristics (Hayes *et al*., 1997; Pietrantonio *et al*., 2000). In the Malpighian tubule of *Drosophila melanogaster*, the drosokinin receptor is expressed in stellate cells and the second messenger is Ca^{2+}, released from intracellular stores (Radford *et al*., 2002). This triggers an intracellular route for chloride secretion towards the Malpighian tubule lumen (O'Donnell *et al*., 1998). In the *Aedes* Malpighian tubule the immediate increase in chloride conductance observed in response to leucokinin is paracellular and not transcellular (Wang *et al*., 1996) and leucokinins are able to increase this paracellular chloride route even when the stellate cells are ablated from the tubule, indicating leucokinins act through the principal cells (Yu & Beyenbach, 2004).

Here we report the cloning of the *A. aegypti* kinin receptor. Based on its cDNA sequence we propose a corrected sequence for the mosquito *An. gambiae* orthologue. The receptor expressed in CHO-K1 cells functionally responds to a leucokinin superagonist and to the three *Aedes* kinins in a dose-dependent manner through the release of intracellular calcium, as expected from a multiligand kinin receptor.

Results

A 3098 bp cDNA (GenBank AY596453) encoding a 584 residue (65.2 kDa) protein was cloned from female *A. aegypti* Malpighian tubule. The nucleotide and predicted amino acid sequences are shown in Fig. 1. The Asp-Arg-His (DRH) motif (residues 136–138, bold in Fig. 1) is located in the second intracellular loop. It is highly conserved among the Class A GPCRs; most often it is present as DRY, sometimes as ERY (Gether, 2000) or ERH but within this class the Arg residue is conserved. This motif is key to the conformational changes leading to receptor activation. It appears that this motif is located in a hydrophilic pocket formed by residues from TM I, TM II and TM VII and that upon agonist binding, protonation of the aspartic (or glutamic) acid residue causes the Arg residue to shift out of the pocket (Gether & Kobilka, 1998). Two cysteines, one present in the first extracellular loop (C112) and the second in the second extracellular loop (C201), are expected to form a disulphide bond to stabilize the receptor (Dixon *et al*., 1987; Schöneberg *et al*., 1999).

Sequence analysis with the TMPred program predicts seven transmembrane regions: TM1 40–64; TM2 76–102; TM3 114–135; TM4 156–176; TM5 217–239; TM6 268–286 and TM7 304–327 (Fig. 1). Analyses with the 'PPSEARCH' program and NetNGlyc 1.0 identified potential ASN-glycosylation sites at asparagine (N) residues 14, 18 and 190; NetPhos 2.0 server identified potential phosphorylation sites at tyrosine (Y) residues 301, 348, 400, 406, 417, 428 and 517; four potential threonine (T) sites at residues 66, 246, 442 and 446, and twenty-three potential serines (S) as phosphorylation sites, with twelve of these residues (Ser 253, 365, 366, 374, 416, 419, 432, 438, 439, 459, 463 and 485) having phosphorylation potential scores of 0.9 or higher on a scale of 0–1. Cysteines 452, 453, 455, 470 and 492 could be palmitoylated, as is common for many GPCRs (Gether, 2000).

Blast searches predict this protein as a GPCR and orthologue of the drosokinin receptor from *D. melanogaster*, and highly similar to the tick myokinin receptor that we previously characterized (Boomi-KR; AF228521) (Fig. 2). Based on our analyses we propose that the *A. gambiae* (Anoga) orthologue receptor gene contains six introns and that the corresponding protein sequence (accession EAA05450) is not correctly predicted. Hydropathy plots

Figure 1. Nucleotide sequence and deduced amino acid sequence of the *A. aegypti* kinin receptor (Aedae-KR) (accession number AY596453). A solid line underlines the seven transmembrane regions characteristic of GPCRs. The start (ATG) and stop codons (TGA) are in bold italics and underlined. Key cysteines (C) are in bold underlined (see text). Residues putatively involved in activation of G protein (DRH) are in bold.

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GTTGAATCCCGGCCGGCATCGCGAACGGTAGCTCTCCAAATCGCTCCAGGAATACCTGATTCG   -775
GATAACGTTTAGCTAGTTGATCTTAATAGTTGAGACATTAAAGTATAAAGAATAGTGCTTGAC   -712
GGGATATAACTTTATAGTTCAAAATTCCTCGCCACCATAGACATCGACCAAAAAAGACGAAGT   -649
TTGTATCATAAATTAAAATTCCCGTCGCAAATTGCCTCGAAATTCAATTTCTCGCTAATCTAG   -586
CAGAGCCAGTGAAAGTTGAAATTTGCGTGCATCACAGTTCAAGTGGTTCCAGCACCCATATTA   -523
GAAATATCCACGAATTGAATCTCGTTTGCTCCGGATCTACCGAAGTGGATTTCAAGTGGTTGG   -460
AGGTGCCGGCAGCAGTTGCTTGCGTATGATTGAGTAGTGGAAACATGGAAAAGGATGTTCTGG   -397
CAATCAGAAGGCAGGCCGGCAAAAAGTGTGTATCCGAAATTTCCAGTGAAAAGCGCATTCCCA   -334
CCGTTAGCTGTCGTATGTATCAACACGGATCATTGCATGGAATGGCAAAGTGATTCTCTTTAA   -271
TTTGGCCGTTTGAAGATTGGACTTCAATGCAGTAGTGGTTTTTGTGCAGTGCAGAAAGTTGGC   -208
AGATGAATGCCGAAGAATGAAATGAAAAACTAATCTGATTGATTCTGATTCAGAAAGTGGTTT   -145
GCTGTTGTGGGAATTGCAGTGCAGATCACTTTGGTTCAGTACATCGAGAAGAATCAACGAACC    -82
TGCAGTTCGACTAGAGAGTGGATTATCTGTTAGTGTTATTTAAATTAAGAGCAGATAAGGAAT    -19
ACCAAATTATCTACAAGA***ATG***CGAGCTGTAGACGGAATCGCCTTCCATTATGCAAACAACAAT     45
                    M  R  A  V  D  G  I  A  F  H  Y  A  N  N  N      15

ACACTGAACGGCAGCGATGTGGAAATAGTCAAAGAGCAGGATGCACTCTACGATGTTCCGGTG     108
 T  L  N  G  S  D  V  E  I  V  K  E  Q  D  A  L  Y  D  V  P  V       36

GGGTTGGTCGTGCTCCTTTCGATTTTCTACGGAACAATCAGTATCATAGCTGTGATAGGCAAT     171
 G  L  V  V  L  L  S  I  F  Y  G  T  I  S  I  I  A  V  I  G  N       57

TCGCTTGTGATATGGATTGTCCTGACGACCAAGCAGATGCAAACCATCACGAACATGTTCATT     234
 S  L  V  I  W  I  V  L  T  T  K  Q  M  Q  T  I  T  N  M  F  I       78

GCGAACCTGGCCTTGGCCGATGTCACCATAGCAGTGTTTGCGATACCCTTCCAGTTCCAGGCT     297
 A  N  L  A  L  A  D  V  T  I  A  V  F  A  I  P  F  Q  F  Q  A       99

GCGGTCCTGCAACGGTGGAATCTGCCGGAGTTCATGTGCCCTTTCTGTCCGTTTGTGCAGCTG     360
 A  V  L  Q  R  W  N  L  P  E  F  M  C  P  F  C  P  F  V  Q  L      120

CTGAGCGTCAACGTGTCGGTTTTTACTTTGACGGCCATAGCCGTTGATCGACACCGGGCCATC     423
 L  S  V  N  V  S  V  F  T  L  T  A  I  A  V  D  R  H  R  A  I      141

ATCAATCCGCTCAGGGCACGTGCTTCGAAAAATATTTCAAAATTTGTCATCAGTGCCATCTGG     486
 I  N  P  L  R  A  R  A  S  K  N  I  S  K  F  V  I  S  A  I  W      162

ATGATGTCGTTCGCACTGGCCGCTCCAACTCTGTTTGCCCTGCGTGTGGTTCCCGTGTCAATC     549
 M  M  S  F  A  L  A  A  P  T  L  F  A  L  R  V  V  P  V  S  I      183

GTTAGCCTGGGCGAAACCAATGAAACCTACATCAATATGACGAAACCATTTTGCCAGGTGGTC     612
 V  S  L  G  E  T  N  E  T  Y  I  N  M  T  K  P  F  C  Q  V  V      204

AATTTTGAAGAGTCGGAGATGCTACTGTATCGATACATCCTGACGTTGGTTCAGTACTTCGTT     675
 N  F  E  E  S  E  M  L  L  Y  R  Y  I  L  T  L  V  Q  Y  F  V      225

CCACTTTGTGTCATCAGTTTCGTGTACATTCAGATGGCACTCAGACTGTGGGGCTCGAAAACT     738
 P  L  C  V  I  S  F  V  Y  I  Q  M  A  L  R  L  W  G  S  K  T      246

CCCGGAAACGCACAGGACTCACGTGATATGACAATGCTGAAAAACAAGAAGAAGGTCATCAAA     801
 P  G  N  A  Q  D  S  R  D  M  T  M  L  K  N  K  K  K  V  I  K      267

ATGTTGATCATCGTGGTGGCCCTGTTCGGAATCTGCTGGTTCCCGCTGCAGCTGTACAATATC     864
 M  L  I  I  V  V  A  L  F  G  I  C  W  F  P  L  Q  L  Y  N  I      288

CTACATGTTACCTGGTCTGAAGTGAACGAGTATCGTTACATTAACATCATTTGGTTTGTCTGT     927
 L  H  V  T  W  S  E  V  N  E  Y  R  Y  I  N  I  I  W  F  V  C      309

GATTGGTTGGCTATGAGCAACAGCTGCTACAACCCGTTTATATATGGTATCTACAATGAAAAG     990
 D  W  L  A  M  S  N  S  C  Y  N  P  F  I  Y  G  I  Y  N  E  K      330
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TTCAAACGAGAATTCCACAAGCGATATCCGTTCCGTGGGCGTAATCAAAGCTACCACCAGGAG   1053
 F  K  R  E  F  H  K  R  Y  P  F  R  G  R  N  Q  S  Y  H  Q  E    351

CAGTTGACCGATAAAACACTCTCGATGTTCACCCGTGTCAGCTCGATCCGGTCCAATTACGCG   1116
 Q  V  T  D  K  T  L  S  M  F  T  R  V  S  S  I  R  S  N  Y  A    372

ACTTCATCGATACGCAACAAGCTCTACACGGGACCGATTGGAGGTGGCAGTGGGAATGGAGGC   1179
 T  S  S  I  R  N  K  L  Y  T  G  P  I  G  G  G  S  G  N  G  G    393

ACCCATGTCGGAAGTGGATACAGTAGCAATGCGTTCTATCAAAACCAAAATTCCCACCATCAG   1242
 T  H  V  G  S  G  Y  S  S  N  A  F  Y  Q  N  Q  N  S  H  H  Q    414

CAATCGTACAAATCTCCTAACACAAATAGTGTCGCTGGTTATCAACGAAACAGCACAACTGAT   1305
 Q  S  Y  K  S  P  N  T  N  S  V  A  G  Y  Q  R  N  S  T  T  D    435

AGGAACAGTAGCAGAAAAACAGCTGCCGGTGCCCCGTGGGATCCCAAGTGTTGTCCATGTCGG   1368
 R  N  S  S  R  K  T  A  A  G  T  P  W  D  P  K  C  C  P  C  R    456

CAAAACTCTACCCGGACTTCCACTGCTGCTGCTTCTGCATGCCCTTATCGGATGCCATTACCT   1431
 Q  N  S  T  R  T  S  T  A  A  A  S  A  C  P  Y  R  M  P  L  P    477

GCAGTGGCCAGCGATGGAGACAGCGGAAGTGAAGGTGGTCCATGTAACAGTGCTGGGGGTGGA   1494
 A  V  A  S  D  G  D  S  G  S  E  G  G  P  C  N  S  A  G  G  G    498

CAAAGTCCCATGATAAACAATGATGAACGACAACTTCTCGGTGCCGATGATAATTATGGAAGC   1557
 Q  S  P  M  I  N  N  D  E  R  Q  L  L  G  A  D  D  N  Y  G  S    519

GCCGCACAGAAACTGGAGGTTATTTCCTTGGACCATCCACATCCGGATAGCGCAGATGACGAG   1620
 A  A  Q  K  L  E  V  I  S  L  D  H  P  H  P  D  S  A  D  D  E    540

AACGGTGTGGCGGAAACGCCACATTCTCGCACTGCTAATGGACAGGAACAGGATGAGAGGTTA   1683
 N  G  V  A  E  T  L  H  S  R  T  A  N  G  Q  E  Q  D  E  R  L    561

CAGCTAACCTCATTTATTTCTTCCGGCAATGGACGACACGAACGGTTTCATTTCCACATTAAC   1746
 Q  L  T  S  F  I  S  S  D  N  G  R  H  E  R  F  H  F  H  I  N    582

AACTTGTAGAATTTGAACCAATTTTACAATTAAGTCCGGTTGTTAATGTTGGTGTGACGATTG   1809
 N  L                                                              584

TTGCGTTTAGAAATATTGGCGCTGAATTGTGACGCGAGCGTCTTCTATGTAGGGAAAGTGTAC   1872
CAGTTATGGCCATAGTGGTTCCTTATTTGACCATATATGAAATTTTGATAACTTTCACACTTT   1935
AATTCATTTTGAATGTTTTAATATCAATATATGTCTTAAATCTAACTACTACAACACACAAAA   1998
ATCTTCAAAATTTGAAGACATGAAATTAATTACGTATGATGAAATAGGGAACCACTATGTCCA   2061
TAACTGGTACACTTACCCTAGTTAATGAGAAAATCTTCTTCATGTGATGTGTAACGATGTCAT   2124
CGAAATAAGGAGTGATGCAAACCAAACTCGAATTACCTATTTATTGAAGTTTTATTCAATATC   2184
CGTATTAACAGAGCCAAAGAGCGCAGAATATAGGGAAGTAAAATATTACTGTATTATGTTGAA   2247
AAAAAAAAAAA                                                       2258
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Figure 1. (*Continued*)

showed that the predicted sequence lacked the N-terminal region, TM1 and C-terminus (data not shown).

The gene likely encodes a protein of 561 amino acids, of which 69 are additional residues at the N-terminus, and has a C-terminus of comparable length to that of the Aedae-KR. The Anoga-KR protein sequence proposed is shown in Fig. 3.

As leucokinins are known to induce contractions in the insect hindgut and the *Aedes* kinins specifically do so in *A. aegypti* (Veenstra *et al.*, 1997), RT-PCR was used for detection of the receptor transcript and confirmation of the identity of the transcript in hindgut. Hindguts completely free of Malpighian tubules were dissected for this purpose, mRNA was isolated and RT-PCR was performed with receptor-specific primers. Sequence analyses of these products confirmed that the same transcript as in the Malpighian tubule is present in the mosquito hindgut, providing

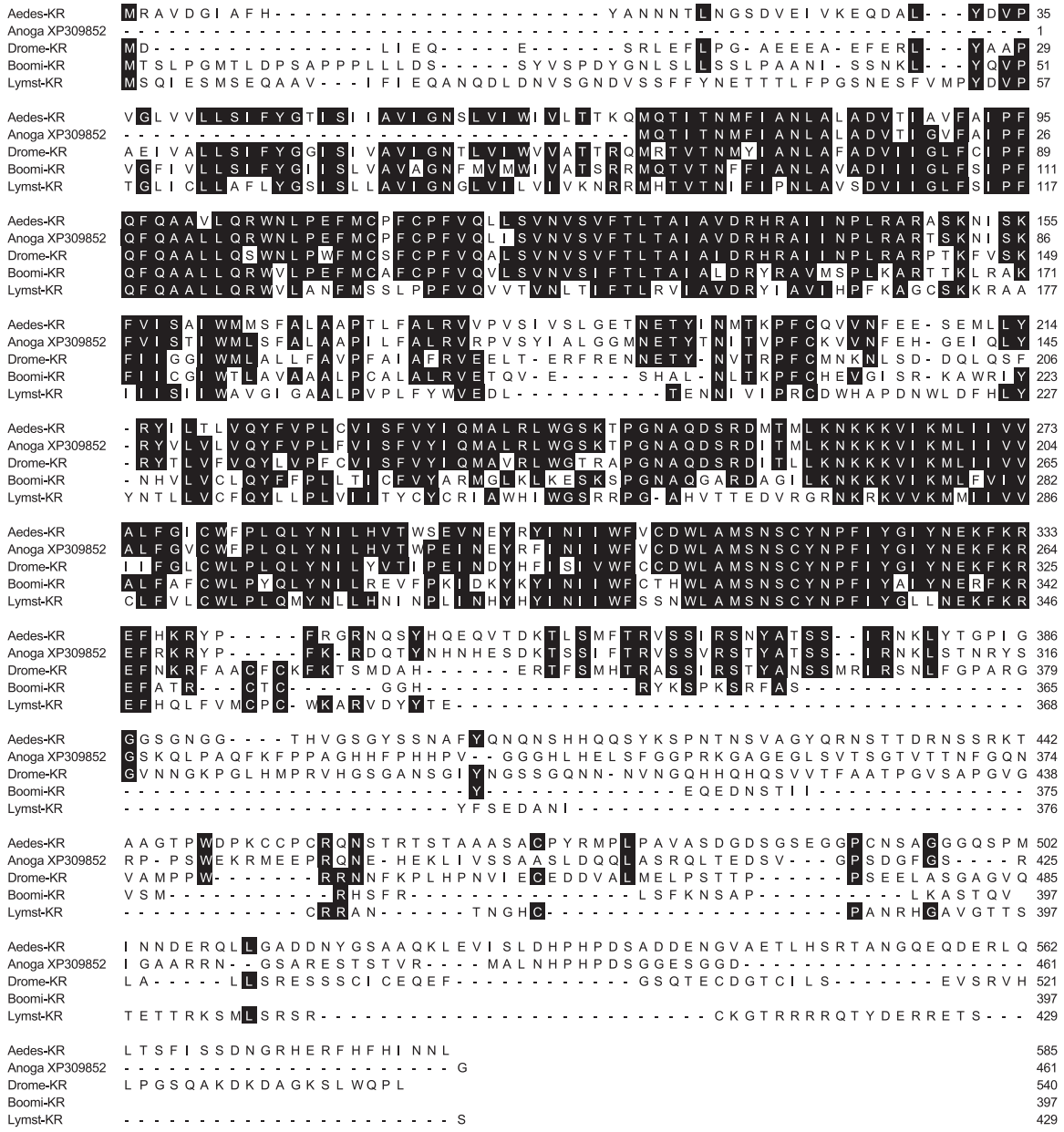

Figure 2. Amino acid sequence alignment of Aedae-KR with other similar receptors. The Aedae-KR is 44.8% similar to the *D. melanogaster* drosokinin receptor, 39.6% similar to the tick *Boophilus microplus* myokinin receptor (Boomi-KR) (Holmes *et al.*, 2000) and 28.1% similar to the *Lymnaea stagnalis* receptor (Cox *et al.*, 1997). The *An. gambiae* sequence Anoga XP309852 (EAA05450) as predicted is incomplete, lacking the first transmembrane region and residues at the C-terminus.

further support to the role of this receptor as an *A. aegypti* kinin receptor (Aedae-KR).

Analysis of receptor function in receptor-expressing CHO-K1 cells (designated E10 cell line) by calcium fluorescence cytometry of individual cells indicated that addition of media without peptides did not produce a significant intracellular calcium response, as expected (Fig. 4A). The Aedae-KR responded in a dose-dependent manner to the addition of a synthetic kinin superagonist analogue (FFF-SWG-NH_2) that has previously been functionally validated in the *A. aegypti* Malpighian tubule and in tick leucokinin receptor research (Fig. 4B; dose–response not shown) (Pietrantonio *et al.*, 2000; Holmes *et al.*, 2003). Most importantly, the receptor-expressing mammalian cells responded to nanomolar concentrations (1 and 10 nM) of the Aedae-K-1 (NSKYVSKQKFYSWG-NH_2), -2 (NPFHAWG-NH_2) and -3 (NNPNVFYPWG-NH_2) by triggering intracellular calcium release, probably from calcium stores (Fig. 4C–E) but not to subnanomolar concentrations (0.1 nM) (Fig. 4C–E). Cells treated with the superagonist (Fig. 4B) showed a

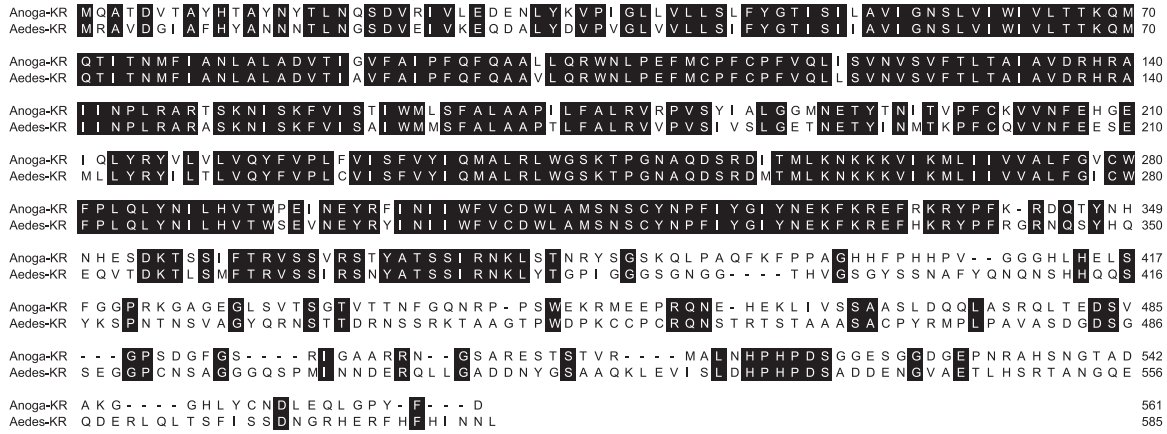

Figure 3. Amino acid sequence alignment of the Aedae-KR (AY596453) and the proposed *An. gambiae* kinin receptor (Anoga-KR; modified EAA05450). This proposed sequence is 59% similar to the *Aedes* receptor.

similar maximum fluorescence response as the kinins (Fig. 4C–E, 10 nM traces), but exhibited a sustained intracellular calcium response with a maximal response lasting for more than 2 min (Fig. 4B). The kinin peptides elicited a maximal response and the individual response of cells decreased slowly after reaching a maximal fluorescence intensity (Fig. 4C–E).

As these results confirmed the activity of the kinins on the E10 cell line, experiments were designed to establish more accurately the order of potency of the three different kinins by estimation of the respective effective concentration (EC_{50}). For this, a bioluminescence assay was developed based on the known principle that intracellular calcium binding to mitochondria-expressed apoaequorin results in light emission in the presence of coelenterazine (Stables *et al*., 1997; Staubli *et al*., 2002). The rank order of potency obtained was Aedae-K-3 > Aedae-K-2 > Aedae-K-1, based on the respective EC_{50} values of Aedae-K-3, 16.04 nM; Aedae-K-2, 26.6 nM and Aedae-K-1, 48.85 nM, which were statistically significantly different ($P < 0.05$) (Fig. 5A–C). Aedae-K1 exhibited not only the highest EC_{50} but also the lowest slope value.

Discussion

Based on structure, sequence analysis and similarities in functionality with leucokinin receptors from the pond snail (Cox *et al*., 1997), the cattle fever tick *Boophilus microplus* (Holmes *et al*., 2000) and *D. melanogaster* (Radford *et al*., 2002) we conclude that we have cloned the leucokinin receptor from the mosquito *A. aegypti*. Furthermore, on the basis of this sequence we proposed a corrected sequence for the orthologue gene of the malaria vector *An. gambiae*.

The possibility of multiple receptors for leucokinins in the mosquito Malpighian tubule was suggested by Veenstra *et al*. (1997). Analysis of the genome of *D. melanogaster* indicates the existence of a single drosokinin receptor and a single ligand, drosokinin. Experiments on *D. melanogaster* adults demonstrated the presence of the drosokinin receptor in stellate cells (Radford *et al*., 2002), where they increase chloride transport through the calcium signalling cascade (O'Donnell *et al*., 1996, 1998). In the mosquito *A. aegypti* Malpighian tubule, electrophysiological and pharmacological studies support the localization of the leucokinin receptor in principal cells (Yu & Beyenbach, 2002). In the cricket Malpighian tubule that does not possess stellate cells, leucokinin increases fluid secretion, the action also mediated by Ca^{2+} (Beyenbach, 2003b).

Contrary to drosokinin action on *Drosophila* Malpighian tubules by which the chloride pathway is intracellular through the stellate cells (O'Donnell *et al*., 1998), it has been shown elsewhere that in *A. aegypti* leucokinin increases fluid secretion by increasing a transepithelial chloride conductance that does not occur through the principal cells (Pannabecker *et al*., 1993) but through an extracellular pathway (Beyenbach, 2003a).

All three *Aedes* kinins increase mosquito hindgut contractions at concentrations of 10^{-9} and 10^{-8} M (Veenstra *et al*., 1997). We were able to verify the transcript presence by cloning the receptor from hindgut mRNA by RT-PCR, from hindguts that were dissected specifically for this purpose without any trace of Malpighian tubules. Therefore, it is possible that this receptor protein is also expressed along the length of the hindgut.

In the isolated Malpighian tubule, Aedae-K-1 and -3 increase fluid secretion whereas Aedae-K-2 depolarizes the transepithelial voltage (V_t) without increasing fluid secretion (Veenstra *et al*., 1997). Interestingly, however, Aedae-K-2 increases fluid secretion *in vivo* (Cady & Hagedorn, 1999b). The percentage of total volume of urine produced by the injection of Aedae-K-1 was 7.5-fold higher than that of females injected with saline after 5 min of

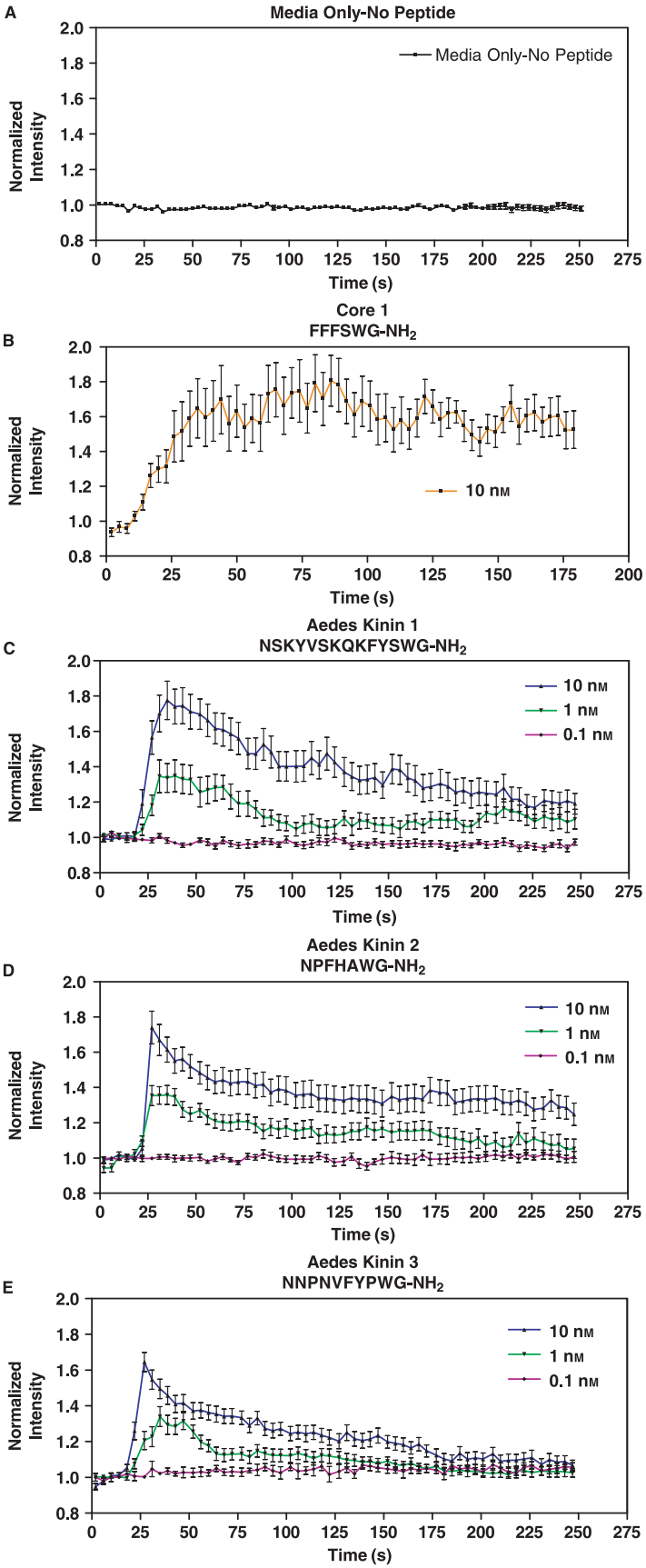

Figure 4. The activation of Aedae-KR by a myokinin superagonist analogue and the three *Aedes* kinins causes intracellular calcium release in CHO-K1 E10 receptor-expressing cells. The graphs show the normalized intracellular fluorescence intensity units (data obtained by confocal fluorescence cytometry) vs. time. Leibovitz's L-15 medium containing 10× final concentration of each peptide (100 μl) was added to cells for a final volume of 1 ml. Fluo-4 AM fluorescence intensity was obtained from image scans recorded from about 13–20 cells every 3 s. After the fifth scan (15–18 s), cells were exposed to peptides and image scans were acquired at the same interval for a total time of 300 s. Traces correspond to the mean fluorescence at each scan measured from cells of two independent replications. (A) Negative control was addition of 100 μl medium without peptides. (B) Response to 10 nM concentration of the kinin superagonist peptide Core1 (FFFSWG-NH_2) used as a positive control (a trace showing a higher response to the 100 nM concentration is not shown). Cells were also independently challenged with the three *Aedes* kinins at physiological range concentrations of 10 nM, 1 nM and 0.1 nM, respectively. Response to (C) Aedae-K-1, (D) Aedae-K-2 and (E) Aedae-K-3. Graphs were produced with the Graphpad Prism 4.0 software.

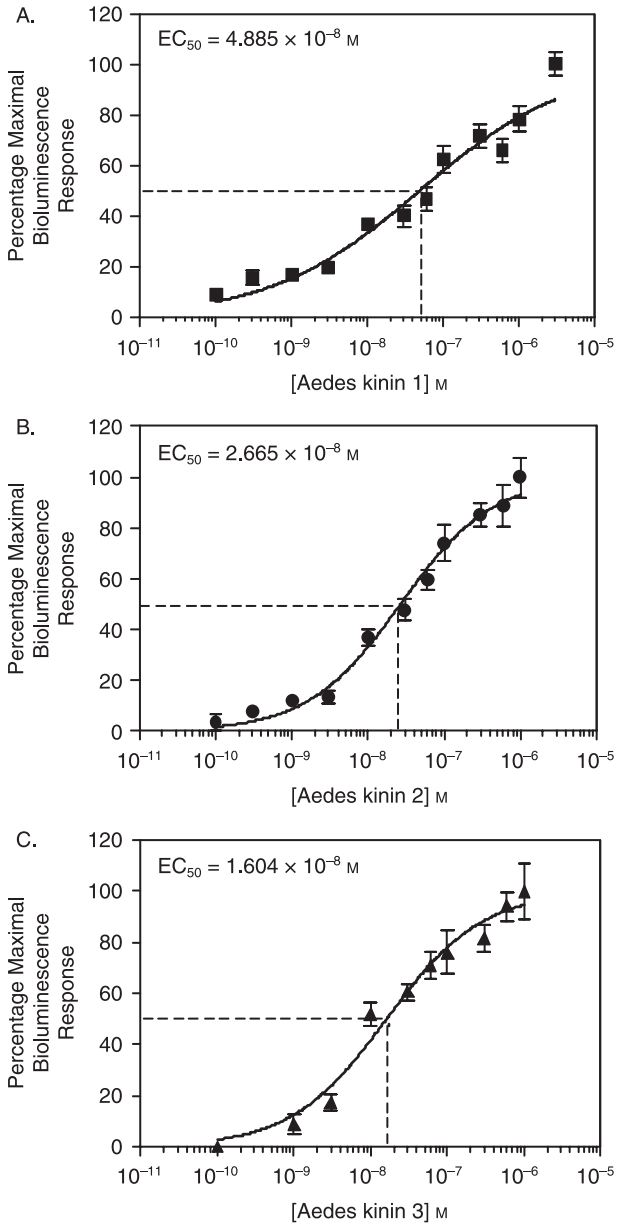

Figure 5. Estimation of *Aedes* kinins effective concentration (EC_{50}) on CHO-K1 E10 cells by a calcium-bioluminescence plate assay. The *y*-axis in the concentration–response curves was obtained from bioluminescence units expressed as a percentage of the maximal response observed for each peptide. Data points represent the average of six replicates obtained during three independent experiments. Bars represent the standard error. (A) Estimation of Aedae-K1 EC_{50} = 48 nM. (B) Estimation of Aedae-K2 EC_{50} = 26 nM. (C) Estimation of Aedae-K3 EC_{50} = 16 nM. EC_{50} Aedae-K3 < EC_{50} Aedae-K2 < EC_{50} Aedae-K1; $P < 0.05$. Statistical analysis and graphs were with the GraphPad Prism 4.0 software.

injection (Cady & Hagedorn, 1999b). As in other insects, the actions of the *Aedes* kinins were specifically verified *in vivo* to be mediated by IP3 in the Malpighian tubule (Cady & Hagedorn, 1999a). In addition, leucokinin VIII, a cockroach peptide, activates a Ca^{2+} conductance in the basolateral membrane of *A. aegypti* Malpighian tubule principal cells that is subsequent to the initial effect of increasing IP_3 production that causes calcium release from intracellular stores (Yu & Beyenbach, 2002). Thus, the *Aedes* receptor was expressed in a heterologous system to determine if myokinins interact with and activate the receptor, by using measurements of intracellular calcium release detected through a calcium-sensitive fluorescent dye in individual cells and by a bioluminescence assay that measures the simultaneous response of thousands of cells. The concentrations of myokinin analogue Core 1 (FFFSWG-NH_2) and Aedae-K-1 (NSKYVSKQKFYSWG-NH_2), -2 (NPFHAWG-NH_2) and -3 (NNPNVFYPWG-NH_2) used in these assays together with the magnitude of the responses obtained correlate well with the reported results *in vitro* with isolated mosquito Malpighian tubules (Veenstra *et al*., 1997), with our similar work with CHO-K1 cells expressing the tick myokinin receptor (Holmes *et al*., 2003), with physiological studies *in vitro* on Malpighian tubule fluid secretion (Veenstra *et al*., 1997) as well as with studies of *Aedes in vivo* urine production (Cady & Hagedorn, 1999b). For example, the synthetic kinin analogue FFFSWG-NH_2 had previously shown to have maximal activity among others in increasing Malpighian tubule transepithelial voltage *in vitro* and in displacing the binding of a leucokinin photolabelling analogue from Malpighian tubule plasma membranes (Pietrantonio *et al*., 2000). It also maximally activated the tick leucokinin receptor when tested using a similar calcium fluorescence assay with CHO-K1 cells expressing this receptor (Holmes *et al*., 2003). The maximum diuretic response of Aedae-K-1 is obtained at 1 μM, and for both Aedae-K-1 and -3 small increases in the rate of fluid secretion were observed when concentrations were increased from 10^{-8} to 10^{-6} M (Veenstra *et al*., 1997). These results are in agreement with the intracellular calcium responses that we report in Fig. 5, where this concentration range elicits responses ranging from 40 to 100% of the maximal bioluminescence response for all kinins. In other studies the physiological role and active concentration of leucokinins has been measured. When injected in *Aedes* females the estimated ED_{50} values per mosquito were 1.53×10^{-4} nmol for Aedae-K-1, 3.56×10^{-4} nmol for Aedae-K-2 and 3.7×10^{-5} nmol for Aedae-K-3 (Cady & Hagedorn, 1999b). In view of the fact that Veenstra *et al*. (1997) found Aedae-K-3 more potent for fluid secretion *in vitro*, it is noteworthy that Cady & Hagedorn (1999b) also found Aedae-K-3 to be more potent in stimulating fluid secretion *in vivo*, although they cautioned that there were not statistically significant differences among the three *Aedes* kinins in ED_{50} for urine production. Our determination of Aedae-K-3 as the most potent kinin in the bioluminescence assay is in agreement with the *in vivo* trends observed by Cady and Hagedorn. Applications of Aedae-K-1 from 5×10^{-6} to 5×10^{-8} M significantly increased IP_3 concentrations in the isolated mosquito Malpighian tubule (Cady & Hagedorn, 1999a). This agrees with our finding

that the EC_{50} for Aedae-K-1 is 4.88×10^{-8} M. The effects of leucokinin VIII from *Leucophaea maderae* assayed on the *A. aegypti* Malpighian tubule are routinely studied at 1 μM concentration (Wang *et al*., 1996). Kinins have been identified in the haemolymph of other insects probably functioning as hormones; the concentration of achetakinin in the cricket ranged between 2.8 to 280 nM depending on feeding status (Chung *et al*., 1994) and in the roach *L. maderae* the leucokinin concentration was between 0.5 and 1.5 nM in the haemolymph (Muren *et al*., 1993). The concentrations of the *Aedes* kinins in the haemolymph of this mosquito are unknown.

In the genome of *A. gambiae* only one receptor (XP309852.1) is predicted for leucokinin and is most similar to the *Aedes* sequence (E value e^{-160}) (Hill *et al*., 2002) (suppl. mat. http://www.sciencemag.org/cgi/content/full/298/5591/176/DC1). There are three *Aedes* kinins encoded by a single cDNA (Veenstra *et al*., 1997) and also three predicted kinins in the *A. gambiae* genome (Riehle *et al*., 2002) (suppl. mat. http://www.sciencemag.org/cgi/content/full/298/5591/172). In summary, the results are consistent with the existence of one leucokinin receptor in *A. aegypti* that responds to the three different endogenous kinins.

Experimental procedures

Insects

Aedes aegypti mosquitoes, Rockefeller strain, were maintained in the laboratory as described (Shapiro & Hagedorn, 1982).

Synthesis of cDNA from female midgut, hindgut and Malpighian tubules

Midgut, Malpighian tubules and hinguts ('digestive system') from sixty non-blood-fed females of *A. aegypti* were dissected into RNA*later*® tissue collection solution (Ambion, Austin, TX, USA) and stored for 5 days at 4 °C. This suspension was diluted 1 : 5 with DEPC-treated water and centrifuged at 10 000 ***g*** to pellet tissue. Poly A^+ RNA was isolated using the Dynabeads® mRNA DIRECT™ Kit (Dynal Biotech Inc., Brown Deer, WI, USA) as per kit instructions unless otherwise specified. Lysis buffer (1 ml) was added to the pellet and tissues were lysed in a glass-on-glass 2 ml homogenizer. Tissue lysate was added to the prewashed beads and incubated for 10 min; the tube was in contact with the magnet for 5 min. Lysate was removed and saved on ice for re-isolation. Beads were washed four times prior to poly A^+ RNA elution. Elution solution was prepared by preheating 18 μl kit elution buffer (10 mM Tris) at 65 °C for 5 min with 1 μl 25× RNA*secure*™ Reagent (Ambion). After removal of the final wash, elution solution (10 μl) was added to beads and incubated at 65 °C for 2 min. Beads were separated with the magnet and the eluant was transferred to a tube on ice. Beads were suspended in lysis buffer for re-extraction of the saved lysate and the same steps were performed; the second extraction eluant volume was 9 μl. The final total volume was 19 μl, to which 1 μl SUPERaseIn™ (Ambion) RNase inhibitor was added. Synthesis of cDNA was with the Marathon cDNA Synthesis kit from Clontech (currently BD Biosciences, San Jose, CA, USA). For first-strand cDNA synthesis, 1 μl cDNA-synthesis primer was added to 4 μl mRNA from DynaBead isolation (twelve digestive system equivalents), and tubes were incubated at 70 °C for 2 min and then on ice for 2 min to denature the mRNA and allow the primer to anneal. Subsequent steps followed the manufacturer's instructions. cDNA synthesis reactions were phenol–chloroform–isoamyl alcohol extracted and back extracted, then ethanol precipitated. Pellets were dried and solubilized in 10 μl of water and stored at −20 °C. Synthesized products were from 500 bp to ~4000 bp as visualized by agarose electrophoresis. Based on kit recommendations and previous gel visualization results, of the final 10 μl of cDNA 1 μl was diluted 1 : 100 with Tricene/EDTA to obtain a working cDNA solution. All cDNAs were stored at −20 °C.

Synthesis of cDNA from Malpighian tubules

Malpighian tubules from fifty non-blood-fed, 2–6-day-old females were dissected and pelleted as above. The pellet was homogenized in 300 μl of lysis buffer. Poly A^+ RNA was isolated using the Dynabeads® mRNA DIRECT™ Kit (Dynal) following the manufacturer's instructions except that the lysate was extracted twice to maximize yield. The final eluant was 20 μl, to which 1 μl of SUPERaseIn™ RNase inhibitor was added. To ensure synthesis of full-length cDNAs, the RNA ligase-mediated (RLM) RACE kit (GeneRacer™, Invitrogen, Carlsbad, CA, USA) was used following the manufacturer's instructions, using 7 μl mRNA (about eighty-four Malpighian tubule equivalents) for the initial dephosphorylation of truncated mRNAs. The reaction was phenol–chloroform extracted followed by ethanol precipitation of the aqueous layer. After air-drying, the pellet was solubilized in 7 μl DEPC-treated water. For removal of the 5′ cap of full-length mRNAs, 1 μl tobacco acid pyrophosphatase (TAP), 1 μl TAP buffer and 1 μl RNase inhibitor were added and the mixture incubated at 37 °C for 1 h. Reactions were phenol–chloroform extracted, ethanol precipitated and solubilized in 7 μl DEPC-treated water. For adaptor ligation, the mRNA sample was added to 0.25 μg (lyophilized) GeneRacer™ RNA-oligo, tubes were mixed, spun down, and incubated at 65 °C for 5 min, followed by addition of 1 μl 10× ligase buffer, 1 μl 10 mM ATP, 1 μl RNase inhibitor and 1 μl T4 RNA ligase (5 U). The tube was incubated at 37 °C for 1 h and placed on ice. Reactions were phenol–chloroform extracted, ethanol precipitated and solubilized in 10 μl DEPC-treated water. For first-strand cDNA synthesis, 1 μl GeneRacer™ oligo-dT primer, and 1 μl (10 mM each) dNTP mix were incubated at 65 °C for 5 min and then on ice for 2 min before adding 4 μl 5× Superscript™ first-strand buffer, 2 μl 100 mM DTT, 1 μl RNase inhibitor, 1 μl Superscript™ II reverse transcriptase (200 U). Reaction was mixed and incubated at 42 °C for 50 min. For enzyme inactivation incubation was at 70 °C for 15 min. One microlitre RNase H (2 U) was added and the mix incubated at 37 °C for 20 min to degrade the mRNA. Storage was at −20 °C.

Cloning of receptor cDNA fragment

Degenerate primers corresponding to putative transmembrane regions six (88F, 5′GAARGTBATCAARATGCTGWTTRTCRTSG3′) and seven (89R, 5′GAAYTCVCGCTTRAABYKYTCRTTVTARATG3′) were designed by identifying conserved regions in leucokinin-like receptors for drosokinin, lymnokinin and *B. microplus* myokinin. DNA amplification was carried out with 1 μl Advantage 2™ TAQ polymerase mix (BD Biosciences) and the above primers (0.08 μM each final concentration) in a total volume of 50 μl using 5 μl double stranded digestive system cDNA (0.06 digestive system

equivalents). Amplification was for forty-five cycles, each at 94 °C, 30 s; 58 °C, 60 s; 72 °C, 120 s. Products were analysed by agarose gel electrophoresis using GelStar® Nucleic Acid Stain (Cambrex, NJ, USA). A 230 bp band was cut and DNA extracted with a QIAquick kit (Qiagen, Valencia, CA, USA). The product was cloned into pCR®2.1-TOPO® vector using a TOPO TA Cloning® Kit (Invitrogen). DNA was prepared from three colonies. The DNA was sequenced from both ends using ABI Big Dye® terminator cycle sequencing (Applied Biosystems Inc., Foster City, CA, USA). Sequencing reactions contained 2 μl ABI Big Dye®, 60 pmol of sequencing primer and 270 ng plasmid template, and were cycled thirty-five times (95 °C, 30 s; 42 °C, 20 s; 60 °C 4 min). All sequencing reactions were cleaned using Micro BioSpin® (Bio- Rad Laboratories, Hercules, CA, USA) chromatography columns, dried, and sent to the Gene Technologies Laboratory (TAMU) for automatic sequencing. Sequence analysis confirmed this 230 bp PCR product as highly similar to the invertebrate leucokinin receptors.

Isolation of receptor cDNA from Malpighian tubule

A specific sense primer P95 (5′GGTTCCCGCTGCAGCTGTACAATATCCTAC3′) for 3′ RACE was designed based on the sequence of the 230 bp product. RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was carried out with 1 μl Advantage 2™ TAQ polymerase mix, specific primer P95 (0.6 μM final concentration) and an adaptor-specific GeneRacer® 3′ antisense primer (0.3 μM) in 50 μl using 1 μl single stranded, adapted cDNA (0.2 Malpighian tubule equivalents). Amplification was for forty-five cycles, each at 94 °C, 30 s; 70 °C, 60 s; 72 °C, 120 s. Products were analysed by agarose electrophoresis and four bands (1600, 950, 510, 480 bp) exceeding the minimum expected size (380 bp) were cut and DNA extracted with the QIAquick kit. The 1600 bp DNA band was cloned into pCR®2.1-TOPO® vector. DNA was prepared from three colonies. Sequencing reactions were assembled as above but with 215 ng plasmid template, and cycled fifty times (95 °C, 30 s; 42 °C, 20 s; 60 °C, 4 min). Sequencing results confirmed this product encompassed the previously described 230 bp product as expected, and also contained an in-frame stop codon and an extensive 3′ UTR. Sequence analysis identified this fragment as the 3′ end of a myokinin-like GPCR. A specific antisense primer was designed downstream of the stop codon for use in 5′ RLM-RACE to clone the complete open reading frame (ORF). This primer (P114, 5′CAGCGCGGAATTCTTCTAAACGCAACAATCG3′) incorporated an *Eco*RI restriction site that resulted in the modification of four nucleotides (underlined). RLM-RACE was carried out as above with primer P114 (0.06 μM) and an adaptor-specific GeneRacer® 5′ Sense Primer in 50 μl using 1 μl single stranded, adapted Malpighian tubule cDNA (0.4 Malpighian tubule equivalents). Amplification was for forty-five cycles (94 °C, 30 s; 70 °C, 60 s; 72 °C, 180 s). After agarose electrophoresis, a ~2800 bp product was cleaned out and cloned into pCR®2.1-TOPO® vector. DNA was prepared from six colonies. Sequencing reactions were assembled as above with 80 pmol of sequencing primer and 200 ng plasmid template and cycled fifty times (95 °C, 30 s; 42 °C, 20 s; 60 °C, 4 min). Blast searches confirmed this sequence as the expected receptor; one clone was chosen for sequencing (KY2800#4) from both directions. Sequence analysis was with the TMPred program for prediction of transmembrane regions and orientation at http://www.ch.embnet.org/software/TMPRED_form.html (Hofmann & Stoffel, 1993). In order to identify potential ASN-glycosylation sites analyses were with the 'PPSEARCH' program (EMBL, http://www.ebi.ac.uk/services/services_tree.html) and NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/) (CBS, Center for Biological Sequence Analysis). NetPhos 2.0 server (at the same CBS site) identified potential protein kinase phosphorylation sites.

A. gambiae *orthologue sequence*

Using the Blast search algorithm at the NCBI, the translated ORF of the *Aedes* receptor sequence was compared with sequences from other species. A gene from *An. gambiae* (gi|31201809|ref|XP_309852.1| ENSANGP00000018201; gi|30178375|gb|EAA05450.2| ENSANGP00000018201) was identified with high similarity to the *Aedes* receptor. Alignment of the protein sequences from both species with DNAStar software indicated that the predicted *An. gambiae* ORF was probably incomplete. The *An. gambiae* genomic DNA sequence corresponding to EAA05450 was obtained from GenBank and analysed using DNAstar software (alignment and hydropathy plot prediction tools), SpliceView intron/exon boundary prediction tool (http://l25.itba.mi.cnr.it/~webgene/wwwspliceview.html), and analysed by Blast comparison with the *A. aegypti* receptor.

Construction of the expression vector

For expression in CHO-K1 cells the clone KY2800#4 was used as template for a PCR using several primers with the purpose of adding a nine amino acid (YPYDVPDYA) haemaglutinin tag (HA-tag) at the N-terminus of the receptor as described (Koller *et al*., 1997; Clark *et al*., 2002; Chen *et al*., 2004). The HA-tag DNA sequence is 5′-TACCCCTACGACGTGCCCGACTACGCC-3′. To add the HA-tag, we designed three overlapping 5′ sense primers for PCR. The first 5′ primer is 5′-AGA***CTCGAG***GCCACC**AT-G**GCCTACCCCTACGACGTG-3′, which contained a *Xho*I restriction site for directional cloning (bold italics), Kozak sequence (Kozak, 1986), an initiation codon (bold) and a partial sequence of the HA-tag (underlined). The second 5′ primer is 5′-GCCTACCCCTACGACGTGCCCGACTACGCCCGA-3′, which contained a partial sequence of HA-tag. The third 5′ primer is 5′-GTGCCCGACTACGCCCGAGCTGTAGACGGAATCGC-3′, which contained partial sequences of the HA-tag and the neuropeptide receptor. The 3′ antisense primer, 5′-CGCAACAATCGT***GGTAC-C***AACATTAACAACCGG- 3′, included a *Kpn*I restriction site (bold italics) for directional cloning. Three 5′ sense primers and one 3′ antisense primer were used in the PCR at the same time. The concentration of two 5′ primers closer to the receptor sequence was diluted to 1% of that of the first 5′ sense primer. PCR conditions were as follows: 10 nM of the first 5′ primer and 100 nM of the 3′ primer; 0.1 nM of the second and third 5′ primers; 176 ng plasmid pCR 2.1 containing the full-length cDNA clone of the neuropeptide receptor; 0.2 mM dNTPs mixture, Advantage 2™ TAQ Polymerase mix and 10× PCR buffer. Parameters were: 94 °C, 3 min; then 94 °C, 30 s; 65 °C, 1 min; 72 °C, 2 min; forty cycles; 72 °C, 5 min. The PCR product was analysed by electrophoresis, cut and cleaned as above and cloned using the TOPO TA cloning kit. The presence of the insert was verified in ten colonies, the DNA of which was digested with *Eco*RI (Promega). Sequencing confirmed that the construct was correctly designed. For cloning into the linearized pcDNA3.1(–) vector (Invitrogen), both vector and PCR product were digested with *Xho*I and *Kpn*I and ligated with T4 DNA ligase (Promega). Competent cells (Top10F′, Invitrogen) were heat-shocked at 42 °C to introduce the plasmid. The positive clone was identified by restriction analysis of recombinants with *Hin*dIII

*and Xba*I, sites that are present in the vector. The expression plasmid, pcDNA3.1/Aedae-KR, was then sequenced.

Peptide synthesis

The insect kinin analogue FFFSWG-NH_2 and *Aedes* kinins-1 (NSKYVSKQKFYSWG-NH_2), -2 (NPFHAWG-NH_2) and -3 (NNPN-VFYPWG-NH_2) were synthesized via solid phase chemistry using Fmoc technology on an ABI 433A peptide synthesizer using Rink-Amide resin (Novabiochem, San Diego, CA, USA) and double-coupling methodology under previously described conditions (Nachman *et al*., 2002). The side chains of amino acids containing functional groups were protected as follows: R (Pmc), H (Trt), S (tBu), Y (tBu) and K (Boc), and purchased from Applied Biosystems, Inc. (Foster City, CA, USA). The crude products were purified on a Waters C18 September Pak cartridge and a Delta Pak C18 reverse-phase column (8 × 100 mm, 15 μm particle size, 100 A pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA, USA) with detection at 214 nm at ambient temperature. Solvent A: 0.1% TFA in water, solvent B: 80% CH_3CN in 0.1% aqueous TFA; conditions: a linear gradient from 20% to 100% B in 40 min, flow rate: 2 ml/min. The purified peptides were quantified via amino acid analysis using the determined nmole value for Phe (Nachman *et al*., 2002) and revealed the expected amino acid ratios. Mass spectrometry analysis was performed on a Kratos Kompact MALDI-TOF MS under previously described conditions (Nachman *et al*., 2002) and observation of the expected parent ions confirmed that the synthesized structures were correct.

Cell culture, transfection and screening of clonal lines

CHO-K1 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown as described (Holmes *et al*., 2003) in a 5% CO_2 humidified incubator at 37 °C. For transfection, CHO-K1 cells were seeded into T-25 tissue culture flasks and allowed to grow overnight (18 h) until 30% confluent. Cells were transfected in serum-free Opti-MEM medium (Life Technologies, Gaithersburg, MD, USA) with the cationic lipid reagent Lipofectin® (Life Technologies) (6 μl) and 2 μg of the expression construct or the empty vector (for negative control) according to the manufacturer's protocol. After 18 h, the lipofectin-containing medium was removed and replaced with F12K (Invitrogen) medium plus 10% fetal bovine serum without antibiotic. After overnight growth, the cells were split into medium without antibiotic as before, and grown for another 18 h. After this, the medium was replaced with the same medium containing 800 μg/ml GENETICIN® (Life Technologies) to generate transfectants by continuous selection for 19 days, which were then maintained in media containing 400 μg/ml GENETICIN®. Cell lines were isolated by serially diluting suspensions of cells across ninety-six-well tissue culture plates beyond a limiting dilution (theoretically less than one cell per well). After 2–3 days of growth, the plates were examined and wells with a single colony that appeared to result from the deposition of a single cell were selected. After single cell colonies had grown to 50–100 cells, they were trypsinized and replated in a ninety-six-well plate and allowed to grow for a further 2–3 days until confluent. Cells lines were transferred to start T-25 cell culture flasks. A single clonal cell line designated CHO-K1 E10 that responded to myokinin (core 1 = FFFSWG-NH_2) challenge with an increase in intracellular calcium was selected for subsequent assays (Fig. 4B). Cells transfected with expression vector only were subjected to the same selection conditions until resistance to GENETICIN® was acquired.

Analysis of peptide activity through intracellular Ca^{2+} measurement

(1) Confocal fluorescence cytometry. This analysis was done as described (Holmes *et al*., 2003). Briefly, about 80 000 cells were seeded in two-well chambered cover-glass slides 48 h prior to assays and grown to 80% confluence. A stock solution of 1 mM fluo-4 AM (Molecular Probes), a Ca^{2+}-sensitive fluorophore, was prepared in dimethyl sulphoxide (DMSO) and diluted with serum- and phenol red-free Leibovitz's L-15 medium with L-glutamine to a 3 μM solution (0.3% final DMSO concentration) for loading cells. Cells were loaded for 1 h with fluo-4 AM after which the monolayer was washed once with medium prior to challenge with peptides. A kinin analogue (FFFSWG-NH_2) was first tried at 1 μM followed by lower concentrations until a response was no longer observed; Aedae-K-1, -2 and -3 were tested at 0.1, 1 and 10 nM. Receptor-expressing cells were also challenged with media only as negative controls. Cells transfected with vector only do not respond to media or peptides as expected (Holmes *et al*., 2003). Agonist-induced changes in intracellular Ca^{2+} were monitored with a Meridian Ultima Confocal Microscope (Meridian Instruments, Okemos, MI, USA) at the Image Analysis Laboratory, Texas A&M University, as described (Holmes *et al*., 2003). Excitation was at 488 nm, and fluorescence emission of 530 nm was collected from individual cells. Initial fluo-4 fluorescence intensity was recorded from about 8–10 selected cells and obtained from at least five image scans, each every 3 s. After the fifth scan, cells were exposed to peptides resolubilized in Leibovitz's L-15 medium, and image scans were acquired at 3 s intervals for a total of 3 min during which peptides were present. The intracellular calcium concentration was expressed as normalized fluorescence intensity units for each cell (traces in Fig. 4). This was calculated as the fluorescence value of each scan divided by the average initial background fluorescence obtained during the first scans (basal fluorescence).

(2) Bioluminescence plate assay. Aequorin plasmid mtAEQ/pcDNA1 was a kind gift from Drs C. J. P. Grimmelikhuijzen and Michael Williamson (University of Copenhagen, Denmark). This plasmid was grown in *Escherichia coli* cells MC1061/P3 (Invitrogen) and was purified with a QIAprep spin miniprep kit (Qiagen Inc.). The protocol for transient transfection was as by Staubli *et al*. (2002). The CHO-K1 E10 cells expressing the *Aedes* kinin receptor were grown in F12K media containing 10% fetal bovine serum and 400 μg/ml GENETICIN® to about 90% confluency in T25 flasks at 37 °C and 5% CO_2. Cells were trypsinized and 2×10^5 cells in 2 ml of media were seeded in each well of six-well tissue culture plates. For a typical assay 2–3 wells were sufficient. Cells were allowed to grow for 24 h in the incubator and typically they were 60% confluent. The medium was removed and replaced with OPTI-MEM medium (Gibco, Invitrogen Co.). For transfection of cells in each well, 96 μl of OPTI-MEM was mixed with 4 μl of the transfection reagent Fugene 6 (Roche Biochemicals) in a microfuge tube. The mixture was incubated for 5 min at room temperature after which 1 μg of aequorin/pcDNA1 plasmid DNA in Tris buffer without EDTA was added and incubated for another 15–20 min at room temperature. This mixture (typically 105–106 μl) was added dropwise to each well with gentle manual shaking, plates were incubated for 4–6 h and then the medium was

changed to F12K medium containing 10% fetal bovine serum without antibiotic. After 24 h cells were trypsinized and transferred to ninty-six-well white thin bottom microtitre plates (Costar 3610) at a density of 40 000 cells/100 μl per well and incubated for 24 h, after which they reach a confluency of 80% that is optimal for performing the bioluminescence assay. To reconstitute the aequorin complex, cells were incubated in 90 μl per well of calcium-free DMEM media (Gibco, Invitrogen Co.) containing 5 μM coelenterazine (Molecular Probes) for 3 h (Stables *et al*., 1997) in the dark at 37 °C and 5% CO_2. Cells were then challenged with different concentrations of peptides (FFFSWG-NH_2, Aedae-K1-3) in a volume of 10 μl (10×) solubilized in calcium-free DMEM media. The assay was performed using the NOVOstar (BMG Labtechnologies) plate reader in bioluminescence mode at room temperature. Light emission (465 nm) was recorded every 2 s over a period of 50 s per well. Concentration–response curves were obtained by nonlinear regression curve fit analysis (sigmoidal dose–response equation with variable slope) using Prism software 4.0 from GraphPad Software Inc. (San Diego, CA, USA). Maximal responses from six individual replicates at each concentration were used for calculations of the EC_{50} values.

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